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(54) Title: <b>EARLY DETECTION OF PROSTATE CANCER (CAP) BY DETERMINING A RATIO INVOLVING PROSTATE SPECIFIC ANTIGEN (PSA) AND HUMAN GLANDULAR KALLIKREIN (hGK-1) CONCENTRATIONS</b>		
(57) Abstract		
<p>The invention relates to a bioaffinity assay of prostate-specific antigen (PSA) comprising the measurement of either the concentration of total PSA (PSA-T), the concentration of free form of PSA (PSA-F) or the concentration of PSA complexed to alpha-1-antichymotrypsin (PSA-ACT), PSA-T being the sum of PSA-F and PSA-ACT. According to the invention, additionally the concentration of human glandular kallikrein (hGK-1) is measured. The concentrations of PSA-T and hGK-1 can be measured in one single assay or in separate assays. The sum of the concentrations of PSA-T and hGK-1 is used to determine the ratio a) PSA-F/(PSA-T + hGK-1) and/or b) PSA-ACT/(PSA-T + hGK-1). Both of these ratios are shown to have an outstanding clinical utility for the discrimination of prostate cancer and benign prostatic hyperplasia.</p>		
<p>The diagram shows three panels: PSA, ACT, and hGK-1. Each panel has a large black shape representing the protein molecule. Inside each shape, there are smaller circles representing epitopes. In the PSA panel, some circles are labeled 'PSA' and others are labeled 'ACT'. In the hGK-1 panel, all circles are labeled 'hGK-1'. Below the panels is a legend:</p> <p>Map defining an epitope accessible on:</p> <ul style="list-style-type: none"><li>○ purified PSA and PSA-ACT complex (PSA-T)</li><li>● purified PSA (PSA-F) but not on PSA-ACT complex</li><li>● neo-hGK-1, purified PSA and PSA-ACT (hGK-1+PSA-T)</li><li>● PSA-ACT complex (PSA-C) and purified ACT</li></ul>		

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**EARLY DETECTION OF PROSTATE CANCER (CAP) BY DETERMINING A RATIO INVOLVING  
PROSTATE SPECIFIC ANTIGEN (PSA) AND HUMAN GLANDULAR KALLIKREIN (hGK-1)  
CONCENTRATIONS**

**FIELD OF THE INVENTION**

This invention relates to a bioaffinity assay for the early detection of prostate cancer (CAP) by employing prostate specific antigen (PSA) and human glandular kallikrein (hGK-5 1).

**INTRODUCTION AND BACKGROUND OF THE INVENTION**

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting 10 the practice, are incorporated by reference.

Prostate-specific antigen (PSA) is an abundant 33 kDa glycoprotein secreted by the epithelial cells of the prostate (Brawer, Acta Oncol 1991; 30:161-8). The concentration of PSA in serum is normally very low but 15 known to frequently increase above normal (i.e. 4 µg/L) in prostate cancer (CAP) patients. Therefore, immunoassays measuring the serum PSA are routinely used to monitor disease progress and relapse of CAP after removal of prostate (Brawer et al. Urology 1989; 5(suppl):11-6, 20 Oesterling J Urol 1991; 145:907-23). However, the specificity of PSA assays for cancer detection is limited by the fact that levels above normal are found also in 30 - 50 % of all patients with benign prostatic hyperplasia (BPH) (Stamey et al. New Engl J Med 1987; 317:909-16, Hudson et al. J Urol 25 1989; 142:1011-7). Serum concentrations of PSA inbetween 4 - 20 µg/L are particularly common in BPH patients and in patients presenting with organ confined, potentially curable CAP. This reduces the utility of the PSA assay in early diagnosis of prostate cancer.

PSA is a serine protease of the glandular kallikrein family and occurs in serum predominantly in complex with the serine protease inhibitor alpha-1-antichymotrypsin (ACT) (Lilja et al. Clin Chem 1991; 37:1618-25). A small amount of PSA circulates in a free, non-complexed form despite a large excess of ACT in serum (Lilja et al. 1991). Most commercial PSA assays detect both free PSA and PSA-ACT complex which constitutes the total PSA fraction (PSA-T). However, all assays do not detect the two forms with equal potency (Stamey et al. J Urol 1994; 152:1-5). This might affect the ability to detect CAP as it has been shown that the proportion of PSA-ACT complex form is higher in serum of CAP patients compared to those with BPH (Stenman et al. Cancer Res 1991; 51:222-6, Christensson et al. J Urol 1993; 150:100-5). This is consistent with the detection of lower free-to-total PSA ratios in CAP than in BPH (Christensson et al. 1993). The reported data also show that specific measurements of the different PSA forms improve the early diagnosis of CAP.

Large amounts of PSA are produced by the glandular epithelium of the human prostate and secreted into the seminal fluid. Low concentrations of PSA are usually found in the general circulation. The serum concentration of PSA is increased in various disease states of the prostate, especially in CAP. PSA measured by immunoassays is the predominant marker in the monitoring of CAP, mainly due to high correlation with tumor volume. The American Cancer Society has recently recommended (Kramer et al. Ann Intern Med 1993; 119:914-23) that determinations of PSA combined with digital rectal examination should be applied to men over 50 years to improve the early detection of CAP. However, the diagnostic potential of PSA measurements in serum is limited by the fact that PSA levels also are elevated in many subjects with BPH. Several improvements have been suggested to enhance the diagnostic specificity of serum PSA determinations.

Experiments designed to determine the sequence of 5' end of PSA mRNA revealed that the prostate expresses another highly similar gene, which was identified as hGK-1 (Chapdelaine et al. FEBS Lett 1988; 236:205-8). Both PSA and hGK-1 have gene structures shared by all tissue kallikreins. Their coding sequences are 85 % similar, their introns 79- 86 % similar and their promoter regions 91 % similar (Lundwall, Biochem Biophys Res Commun 1989; 161:1151-9, Riegman et al. Biochem Biophys Commun 1989; 159:95-102, Scheidlich et al. Clin Exp Pharmacol Physiol 1988; 15:339-44). The similarity between PSA and the predicted hGK-1 protein is very pronounced and higher than that between either PSA and KLK1 (kallikrein 1) or hGK-1 and KLK1. The prostatic expression of hGK-1 has been shown to be restricted to the secretory epithelial cells, and may be 10 - 50 % lower than that of PSA as shown by the prostate levels of hGK-1 and PSA mRNA, respectively (Young et al. Biochemistry 1992; 31:818-24). So far, the prostate is the only identified location of hGK-1 gene expression. Thus, although the hGK-1 gene is expressed in the same prostatic epithelial cell as the PSA gene, the predicted hGK-1 protein remains to be characterized in vivo. However, the coding sequences of hGK-1 predict a 261 amino acid preprotein which would give rise to a mature glycoprotein of 237 residues.

PSA is a 33 kDa glycoprotein and the mature protein consists of 237 amino acids (Lundwall & Lilja, FEBA Lett 1987; 214:317-22, Riegman et al. Biochem Biophys Res Commun 1988; 155:181-8, Henttu et al. Biochem Biophys Res Commun 1989; 160:903-10). The amino acid sequence of mature PSA has also been confirmed by analysis of purified protein (Schaller et al. Eur J Biochem 1987; 170:111-20, Watt et al. Proc Natl Acad Sci USA 1986; 83:3166-70). The hGK-1 amino acid sequence predicts one asparagine-linked glycosylation site, but at a different position when compared to PSA. The hGK-1 protein remains to be isolated and characterized.

Certain structural features of PSA and the putative hGK-1 protein are shown in Figure 1 (Henttu et al. Ann Med 1994; 26:165-71). It is likely that the three-dimensional structures of PSA and hGK-1 are very similar as both

- 5 proteins retain the 10 cystein residues involved in the folding of kallikrein. There are six regions in PSA and hGK-1 polypeptide chains where 15 consecutive amino acids are identical. Therefore, it is very likely that PSA and hGK-1 share antigenic epitopes that are exposed in both  
10 proteins, as these regions also span hydrophilic regions in the proteins (Henttu et al. 1989).

In the present invention we have produced recombinant hGK-1 and PSA in vitro. The structures of the expressed proteins have been confirmed. Epitope maps for both proteins have

- 15 been constructed employing a wide selection of monoclonal antibodies previously raised and characterized against PSA.

Monoclonal antibodies were identified that share common antigenic epitopes on both PSA and hGK-1 in addition to monoclonal antibodies that recognize epitopes on PSA only.

- 20 The identified monoclonal antibodies were used to design assays to measure total PSA + hGK-1 and total PSA alone.

Serum samples from CAP and BPH patients were used to measure the concentrations of free PSA, total PSA, total PSA + hGK-1 and PSA-ACT. A clinical validation of the

- 25 results was performed. Moreover, a separate assay was designed to measure only the hGK-1 concentration in serum samples. This is achieved by preincubating the sample with a PSA-specific monoclonal antibody to prevent PSA to be detected by an assay detecting both PSA and hGK-1.

- 30 Proteolytically active PSA slowly forms stable complexes in vitro with alpha-1-antichymotrypsin (ACT), alpha-2-macroglobulin, protein C inhibitor and pregnancy zone protein. In 1991 (Lilja et al. 1991) it was shown that PSA complexed to ACT is the major form of PSA in serum. An  
35 immunometric assay specific for the free non-complexed PSA was used to estimate that on average 15 - 20 % of the total

- PSA concentration occurs in this form. Two-site immunometric assays of PSA-T (i.e. total PSA), PSA-ACT (i.e. PSA complexed to ACT) and PSA-F (i.e. free PSA) reported in this investigation were later used to
- 5 demonstrate (Christensson et al. 1993), Lilja et al. International pat appl publ no WO 92/01936) that the ratios of PSA-F/PSA-T, being smaller in CAP than in BPH, or PSA-ACT/PSA-T, being greater in CAP than in BPH, improved the discrimination between the two groups of patients.
- 10 Independent findings have later confirmed the original observation of the increased discrimination between CAP and BPH obtained by the measurement of the ratio of PSA-F/PSA-T. When, according to the present invention, the measurement of hGK-1 in addition to the different forms of
- 15 PSA became possible, serum samples from CAP and BPH patients were analyzed in regard to the usefulness of hGK-1 or hGK-1 in combination with different forms of PSA in the discrimination of these two patient groups.

SUMMARY OF THE INVENTION.

- 20 According to the present invention the occurrence and concentration of the hGK-1 protein has for the first time been measured in human serum. Due to the specificity of the selected monoclonal antibodies, two-site immunometric assays were used to measure either the concentration of
- 25 both hGK-1 + PSA-T or that of PSA-T only. Another two-site immunometric assay was used to measure the concentration of PSA-F. A fourth two-site immunometric assay was used to measure the concentration of PSA-ACT (the PSA complexed to alpha-1-antichymotrypsin). An early detection of CAP and an
- 30 efficient discrimination between CAP and BPH is obtained according to this invention by the determination of either the ratio PSA-F/(PSA-T + hGK-1) or the ratio PSA-ACT/(PSA-T + hGK-1). The bioaffinity assay can e.g. be carried out as an immunoassay based on the use of
- 35 antibodies. Alternatively, the bioaffinity assay can be performed by using genetically constructed bioaffinity

components (i.e. antibody fragments) specific for the antigen(s) in question (Marks et al., J Biol Chem 1992; 267:16007-10; Winter et al., Annu Rev Immunol 1994; 12:433-55; Owens and Young, J Immunol Methods 1994; 168:149-165).

- 5 The sum PSA-T + hGK-1 can be measured simultaneously using antibodies or antibody fragments that recognize common isotopes on both PSA and hGK-1. Alternatively, the amounts of PSA-T and hGK-1 are measured separately after which the sum is calculated. The clinical utility of the new ratios  
10 (i.e. PSA-F/(PSA-T + hGK-1), or PSA-ACT/(PSA-T + hGK-1) for the discrimination between CAP and BPH is similar or possibly increased when compared to the usefulness in measurements of the ratio PSA-F/PSA-T or PSA-ACT-/PSA-T, respectively. Therefore, the ratio PSA-F/(PSA-T + hGK-1) as  
15 determined according to the invention is also smaller in CAP than in BPH while the ratio PSA-ACT/(PSA-T + hGK-1) is greater in CAP than in BPH.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 shows certain structural features of PSA and hGK-1 enzymes. Amino acids that differ between the mature PSA and hGK-1 polypeptide chains are shown by black circles in the hGK-1 figure. The positions of the five disulphide bonds (shown by the letter C within a circle) and the positions of the active site residues (letters within circles) are based on the typical structure of serine proteases. The active site residues considered to be involved in determining the substrate specificities of PSA (i.e. serine 183) and hGK-1 (i.e. aspartate 183) are indicated by a letter within a box. The glycosylation sites (i.e. 20 asparagine 45 in PSA and asparagine 78 in hGK-1) are indicated by branched structures. The figure is modified from Bilhartz et al., Urology 1991; 38:95-102 and no attempt has been made to illustrate the three-dimensional folding of the polypeptide chains.  
25  
30

Figure 2 illustrates an ECL Western blot detection of the proteins with 0.1 µg/ml of B1 + B6 monoclonal antibodies. The figure is drawn according to the original experimental data.

- 5 Lane 1: purified PSA from seminal plasma
- Lane 2: recombinant PSA
- Lane 3: recombinant hGK-1

Figure 3 shows the epitope maps for PSA, PSA-ACT and hGK-1. Overlapping circles indicate no sandwich formation.

10 Touching circles indicate interfering sandwich formation. Separate circles indicate independent epitopes.

Figures 4A to 4C illustrate the bioaffinity assay (immunoassay) design for specific detection of hGK-1 and its complexes:

15 Figure 4A shows the blocking reaction. The sample containing PSA, hGK-1 and their complexes are reacted with an excess of a Mab A5 specifically recognizing PSA alone. This serves to totally block PSA from participating in the subsequent immunoreactions. The blocking step is carried  
20 out in a well coated with streptavidin.

Figure 4B shows the specific capture of hGK-1. A biotinylated Mab B6, equally well recognizing PSA and hGK-1 (and their complexes), is added to capture hGK-1 to the streptavidin surface. Reaction between Mab B6 and PSA is  
25 blocked by the presence of Mab A5. After completed incubation, the wells are washed. This completely removes PSA and its complexes.

Figure 4C shows the detection of hGK-1. An excess of labelled antibody Mab B1 (recognizing both PSA and hGK-1 and their complexes) is added to detect the hGK-1 bound to the streptavidin surface through Mab B6.

Figure 5 shows the receiver operation characteristics (ROC)

for the discrimination of male subjects without CAP (BPH + Normal) from patients with CAP using the ratios obtained for the four different assay combinations

PSA-F/(hGK-1 + PSA-T); PSA-F/PSA-T; PSA-C/(hGK-1 + PSA-T)

5 and PSA-C/PSA-T. PSA-C means the same as PSA-complexed ACT, i.e. PSA-ACT. The areas under the curves (AUC) were calculated and compared. Number of samples: BPH + Normal = 57; CAP = 32. PSA-T cut-off 1 - 20 µg/L.

## 10 DETAILED DESCRIPTION OF THE INVENTION

### 1. Production of recombinant proteins

#### Materials

The DNA coding for PSA was derived from a PSA cDNA clone in pGEM (Lundwall and Lilja, 1987). The hGK-1 cDNA was cloned

15 as described by Young et al. 1992 and ligated into the plasmid vector puc 19. The vectors pSFV1, pSFV3, pSFV3-lacZ and pSFV-Helper2 were a gift from P. Liljeström (Liljeström and Garoff, Biotechnology 1991; 9:1356-61). The PCR primers 5'-PSAV3: 5'-TAT GGA TCC CAT GTG GGT CCC GGT TGT C-3', 3'-  
20 PSA: 3'-TAT GGA TCC TTA GGG GTT GGC CAC GAT GGT G-5', 5'-hGK-1V3: 5'-TAT GGA TCC CAT GTG GGA CCT GGT TCT C-3', 3'-hGK-1: 3'-TAT GGA TTC TTA GGG GTT GGC TGC GAT GGT-5' and the sequencing primers SP1 and SP2 (Liljeström and Garoff, 1991), 5'-PSAM: 5'-AGC CTC CTG AAG AAT CG-3', 3'-PSAM: 3'-  
25 TGA CCT TCA CAG CAT CCG-5', 5'-hGK1M: 5'-CCT TAG ACC AGA TGA AG-3' and 3'-hGK1M: 3'-GAC CTT CAC AAC ATC TG-5' were synthesized on an Applied Biosystems oligo-nucleotide synthesizer. Molecular biology reagents and enzymes were obtained from Appligene, Boehringer Mannheim, Pharmacia  
30 Biotech or Promega. Sequencing was done with PRISM Sequenase Terminator (Applied Biosystems, Foster City, CA). The western blotting reagents were from Amersham (Buckinghamshire, England).

**Equipment**

Agarose gel electrophoresis was run with an equipment from Appligene. The PCR was run with a MiniCycler (SDS, Falkenberg, Sweden). The sequence gels were run and  
5 analyzed with the 373 DNA Sequencer from Applied Biosystems. The electroporations were done with Gene Pulser (Bio-Rad, Richmond, CA). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was run with the Mini PROTEAN II system from BioRad. Western blotting of  
10 proteins was done with Semi Dry Blotter II from Kem En Tec.

**Construction of pSFV3-PSA and pSFV3-hGK1**

The work with DNA was done with conventional techniques described by Sambrook et al. A Laboratory Manual, 1989 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor,  
15 N.Y. if not otherwise stated. The bacterial cells used in the work were E. coli DH5alpha. Competent cells were prepared as described in BioRads Gene Pulser manual. The DNA coding for preproPSA was amplified with polymerase chain reaction (PCR) from pGEM-PSA with primers 5'-PSAV3 and  
20 3'-PSA and the DNA coding for preprohGK1 from pUC19-hGK1 with primers 5'-hGK1V3 and 3'-hGK1 using Pfu DNA polymerase (Stratagene, LaJolla, CA). The reaction consisted first of 5 cycles of denaturation at 94°C for 50 seconds, annealing at 58°C for 1 minute, and polymerization at 72°C for 2  
25 minutes followed by 25 subsequent cycles of denaturation at 94°C for 50 seconds, annealing at 64°C for 1 minute and polymerization at 72°C for 2 minutes. All primers contained a BamH1 restriction site which was used to ligate the fragments into the vectors. After cutting the fragments  
30 with BamH1 they were isolated from an agarose gel with JetSorb (Genomed GmbH, Bad Oeynhausen, Germany), purified and ligated into puc18. The fragments were cut out of puc18 with BamH1, purified as before and then ligated into pSFV3. The nucleotide sequences of the final constructs were  
35 verified by DNA sequencing. The primers used in the

sequence reactions were SP1, SP2, 5'-PSAM, 3'-PSAM, 5'-hGK1M and 3'-hGK1M.

#### Expression of recombinant PSA and hGK-1

Protein expression was done with Semliki Forest Virus  
5 expression system (Berglund et al. Biotechnology 1993;  
11:916-920). The protocols used were from the SFV/Helper 2  
gene expression system users' manual (Life Technologies,  
Gibco BRL). The vectors were first linearized with SpeI.  
The mRNA was generated in a total volume of 50 µl with 30  
10 units SP6 polymerase from 1.5 µg of the linearized vector  
in a SP6 buffer containing additionally 1 mM ATP, CTP, and  
UTP, 0.5 mM GTP, 1 mM m7G(5')ppp(5')G and 50 units RNasin.  
The reaction mixture was incubated for 2 hours at 37°C.  
Half of the mRNA was transferred to BHK-21 cells by  
15 electroporation. The BHK-21 cells were grown in MEM (Gibco  
BRL) supplemented with 2 mM glutamine, 100 U/ml penicillin,  
100 µg/ml streptomycin, non essential amino acids (ICN  
Biomedicals, Costa Mesa, CA) and 5% fetal bovine serum. The  
cells were detatched with Trypsin-EDTA, washed with PBS  
20 without Mg<sup>2+</sup> and Ca<sup>2+</sup> and suspended to a concentration of  
about 10x7 cells per ml. The electroporation was done by  
pulsing twice with 850 V/25 µF in a 0.4 cm cuvette using  
0.8 ml of the cell mixture. After the electroporation the  
cells were suspended in 20 ml of culture medium and  
25 transferred to a culture bottle. The supernatant containing  
the expressed proteins was collected after 48 or 72 hours.  
The function of the expression system in our hands was  
checked by doing all the steps of the expression of PSA and  
hGK1 in parallel with the expression of β-galactosidase  
30 with pSFV3-lacZ (Liljeström & Garoff, 1991).

#### Electrophoresis and immunoblotting

The size separation of the proteins was done with SDS-PAGE  
run as described in the Mini-PROTEAN electrophoresis cell  
instruction using 12 % polyacrylamide gels. The size of the

expressed proteins was verified with molecular mass markers for SDS-PAGE (Pharmacia, Uppsala, Sweden) and by ECL-western blotting (Amersham, Buckinghamshire, England) using the protocol suggested by the manufacturer. The detection 5 was done with a 0.1 µg/ml mixture of biotinylated monoclonal antibody B1 and B6 and streptavidin-horseradish peroxidase.

Verification of the protein produced

The nucleotide sequences coding for the prepro-form of the 10 proteins and the orientation of the DNA inserted in the pSFV3 vectors were verified by nucleotide sequencing to validate that no base substitutions had occurred during the construction of the vectors. The pSFV3-PSA codes for a 261 aa preproPSA and pSFV3-hGK1 codes for a 261 aa preprohGK1. 15 Both constructs contained a sequence coding for an extra Met-Asp-Pro in the beginning of the proteins. The nucleotide sequence coding for hGK-1 differs from the genomic sequence reported earlier (Schedlich et al., DNA 1987; 6:429-437) at nucleotides 600 (G->A) and 663 (G->T) 20 (relative to the initiation codon). These changes are different from those reported earlier in cDNA sequences (Riegman et al., Molecular and Cellular Endocrinology 1991; 76:181-190; Young et al., 1992). The changes do not affect the amino acid sequence that is identical to that 25 previously reported (Schedlich et al., 1987).

Size of recombinant PSA and hGK1

The size of the proteins was determined by western blot after SDS-PAGE using monoclonal antibodies B1 and B6. The size of recombinant PSA and hGK-1 was identical to that of 30 PSA purified from seminal plasma (Figure 2).

## 2. Epitope mapping

Epitope mapping studies using previously described procedures (Pettersson et al. Clin Chem in press) were carried out by the analysis of the immunodetecting capacity 5 of every possible combination of the available monoclonal anti PSA antibodies: one Mab being immobilized to the microtitration well and the other labelled with Eu-chelate, by using as antigen either purified PSA (isolated from seminal fluid) or recombinantly produced PSA or hGK-1.

10 After a first qualitative test (no binding versus detectable binding) affinity constants were determined according to a previously reported protocol (Pettersson et al. Clin Chem in press) for the PSA-Mabs recognizing rhGK-1. The data are compiled in Table I. The respective epitope 15 maps for the monoclonal antibodies recognizing epitopes on PSA, PSA-ACT and hGK-1 are shown in Figure 3. Six of the tested monoclonal anti-PSA antibodies (B1, B2, B3, B4, B6 and B7) were found to be equally reactive with rhGK-1, rPSA and PSA purified from seminal fluid. One additional 20 monoclonal antibody (B5) also recognized rhGK-1 but the affinity for hGK-1 was significantly lower than that for PSA. In total, seven of the tested monoclonal anti-PSA antibodies (A1-A7) recognized epitopes that were unique to PSA and not shared with rhGK-1. These epitopes are exposed 25 on both free PSA and on PSA-ACT, five (C1-C5) recognized epitopes unique to PSA that were exposed on free PSA alone, and seven (B1-B7) recognized epitopes available on both free PSA and PSA-ACT that were common to hGK-1. The specificities of the monoclonal antibodies are given in 30 Table 1.

**Table 1. Affinity constants and specificity of Mabs**

antibody	affinity constant			specificity
	PSA	PSA-ACT	hGK-1	
A1	$3 \times 10^9$	$4 \times 10^8$		PSA-T
A2	$2 \times 10^9$	n.d.		•
A3	$2 \times 10^7$	$1 \times 10^7$		•
A4	$4 \times 10^9$	n.d.		•
A5	$7 \times 10^8$	$4 \times 10^8$		•
A6	$6 \times 10^7$	$3 \times 10^7$		•
A7	$8 \times 10^8$	$7 \times 10^8$		•
B1	$4 \times 10^{10}$	$2 \times 10^{10}$	$1 \times 10^{10}$	hGK-1 + PSA-T
B2	$5 \times 10^8$	$5 \times 10^8$	$2 \times 10^9$	•
B3	$6 \times 10^8$	n.d.	$7 \times 10^8$	•
B4	$5 \times 10^8$	$5 \times 10^8$	$2 \times 10^9$	•
B5	$2 \times 10^{10}$	$8 \times 10^9$	$3 \times 10^9$	•
B6	$3 \times 10^9$	$2 \times 10^9$	$3 \times 10^8$	•
B7	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$	•
C1	$3 \times 10^{10}$			PSA-F
C2	$1 \times 10^9$			•
C3	$2 \times 10^8$			•
C4	$5 \times 10^9$			•
C5	$2 \times 10^8$			•
D1		$1 \times 10^9$		PSA-C and ACT

3. Assays for specific determination of PSA-F, PSA-T, PSA-T + hGK-1 and PSA-ACT using selected monoclonal antibody combinations

- 5 Based on the information obtained from the PSA and hGK-1 epitope maps and the determinations of the affinity constants, two immunofluorometric assays were constructed. The combination of monoclonal antibody B1 on the solid phase with monoclonal antibody B6 as a tracer co-detects PSA and hGK-1 (PSA-T + hGK-1). The combination of
- 10

monoclonal antibody A1 on the solid phase with monoclonal antibody B1 as a tracer provides specific measurements of both free PSA and PSA-ACT (PSA-T), but does not co-detect hGK-1. An additional assay was optimized using monoclonal 5 antibody B1 on the solid phase and monoclonal antibody C2 as tracer for the specific measurement of free PSA (PSA-F). This assay did not co-detect hGK-1. It was not possible to design an assay that selectively detected the free form of PSA that also co-detected hGK-1, since all monoclonal 10 antibodies specifically detecting the free form of PSA were unable to recognize hGK-1. Moreover separate assay was made to measure only PSA-ACT by using monoclonal antibody B1 on the solid phase and monoclonal antibody D1 as tracer. The assays were set up according to previously published 15 procedures (Lilja et al. 1991, Pettersson et al. Clin Chem in press).

An additional assay was designed for the specific measurement of hGK-1. As no monoclonal antibody was available that would directly and exclusively recognize 20 hGK-1 but not PSA, this assay employs a separate step to totally and specifically block the recognition of both the free form of PSA and PSA-ACT. This is achieved by addition of an excess of monoclonal antibody A5, which recognizes both the free form of PSA and PSA-ACT but does not cross-react with hGK-1. This prevents PSA from reacting in the 25 subsequent immunofluorometric assay (biotinylated monoclonal B6 as catcher antibody /labeled monoclonal B1 as tracer antibody) which without the blocking step measures equally well PSA and hGK-1. This is due to the overlapping between the A5 and B6 epitopes (Figure 3). An example of the detailed performance of this assay is 30 described in Figure 4A to 4C. A control assay to hGK-1 was performed in an identical way, but by omitting the step employing the PSA blocking monoclonal antibody A5: Assay 35 PSA + hGK-1.

4. Discrimination between clinical BPH and CAP by the measurement of the ratio PSA-F/PSA-T, PSA-F/(PSA-T + hGK-1) or PSA-ACT/(PSA-T + hGK-1).

57 serum samples from normal male subjects and patients  
5 with BPH and no evidence of CAP and 32 pre-treatment  
samples from patients with documented CAP were tested with  
assays for PSA-F, PSA-T, PSA-T + hGK-1 and PSA-ACT (Table  
2). The respective ratios: PSA-F/PSA-T; PSA-F/(PSA-T + hGK-  
1); PSA-ACT/PSA-T; and  
10 PSA-ACT/(PSA-T + hGK-1) were calculated for all the  
samples. Receiver operation characteristics (ROC) analyses  
were calculated for the discrimination of BPH from CAP  
using the ratios obtained for the four different assay  
combinations mentioned above (Figure 5). The areas under  
15 the curve using ratio PSA-F/PSA-T was 0.80, using ratio  
PSA-F/(PSA-T + hGK-1) it was 0.83, using ratio  
PSA-ACT/PSA-T it was 0.69 and using ratio  
PSA-ACT/(PSA-T + hGK-1) it was 0.63, respectively. Thus the  
ratio between PSA-F/(PSA-T + hGK-1) gave the best  
20 performance while the ratio between PSA-ACT/(PSA-T + hGK-1)  
gave the lowest discrimination between the patient groups.  
According to the results it is obvious that an assay for  
the measurement of PSA-T + hGK-1 is useful in the  
discrimination of BPH from CAP and in early detection of  
25 CAP when used in combination with the measurement of PSA-F  
or PSA-ACT to calculate the ratios to be used.

**Table 2. Assays for PSA-F, PSA complexed to ACT (=PSA-C), PSA-T and PSA-T + hGK-1. PSA-T cut off level 1 - 20 µg.**

**NORMALS**

µg/L sample	PSA-T+hGK-1 B1/B6	PSA-T A1/B6	PSA-F C2/B1	PSA-C B1/D1
6	1.71	1.84	0.30	1.74
27	3.05	3.28	0.39	3.51
28	2.37	2.46	0.71	1.85
39	4.91	5.44	0.90	4.38
41	1.59	1.83	0.32	1.08
43	1.08	1.44	0.19	0.71
47	1.89	2.35	0.19	1.61
50	1.96	2.20	0.36	1.82
56	7.16	7.62	2.00	5.99
63	1.11	1.38	0.06	0.78
67	2.05	2.29	0.11	1.70
68	4.45	5.35	0.80	3.61
73	2.50	3.08	0.15	2.20
76	2.12	2.78	0.43	1.61
86	1.48	1.79	0.21	1.15
88	2.92	3.78	0.38	2.32
89	1.00	1.52	0.24	0.74
90	2.64	3.50	0.46	1.77
111	4.46	4.94	1.01	3.20

n= 19

**BPH**

µg/L sample	PSA-T+hGK-1 B1/B6	PSA-T A1/B6	PSA-F C2/B1	PSA-C B1/D1
3	1.46	1.54	0.37	1.39
7	10.05	10.62	1.79	9.96
8	1.66	1.62	0.38	1.41
13	18.41	20.22	3.22	19.67
14	16.12	15.85	3.90	15.15
24	2.95	3.77	0.72	2.07
30	7.21	7.47	1.64	6.69
36	7.71	7.87	3.42	5.05
44	3.18	3.64	0.43	2.99
57	3.97	4.70	0.38	3.30
58	15.00	14.57	3.66	13.59
72	14.30	14.03	1.46	13.96
75	1.53	1.98	0.29	1.77
77	3.13	4.21	0.42	2.36
79	5.17	6.53	0.96	4.02
82	4.54	4.56	0.98	2.93
85	1.29	1.92	0.32	0.91
87	11.28	14.30	1.75	8.26
92	4.15	4.08	0.51	3.02
95	2.28	2.63	0.37	1.62
96	2.18	2.88	0.22	1.61
97	2.78	3.71	0.27	1.95
101	6.04	7.02	0.62	5.09
105	3.92	4.39	0.46	3.20
106	5.99	7.00	1.35	4.56

cont.

continuation of Table 2:

107	10.75	12.19	2.63	7.51
109	1.07	1.31	0.12	0.95
114	9.06	8.86	0.75	7.22
115	9.80	10.40	3.42	5.74
116	4.96	5.65	1.23	3.35
120	13.47	15.05	1.20	11.53
124	1.02	1.32	0.31	0.69
135	12.77	15.62	2.05	10.83
136	2.42	3.30	0.15	2.46
137	7.83	9.27	1.15	6.66
144	19.22	20.48	1.99	16.06
145	14.35	16.67	4.53	9.72
210	7.86	8.91	0.35	6.71

n= 38

## CAP

ug/L sample	PSA-T+hGK-1 B1/B6	PSA-T A1/B6	PSA-F C2/B1	PSA-C B1/D1
225	3.10	3.56	0.15	2.80
1	9.57	10.42	1.23	10.61
62	16.86	16.43	1.40	17.02
142	19.03	20.86	1.02	16.54
183	19.76	21.91	1.28	17.95
326	10.75	11.07	0.40	8.68
99	16.54	15.72	2.05	11.22
112	12.50	14.45	1.63	10.61
118	3.68	4.23	0.89	2.30
2	1.89	1.77	0.18	1.28
15	4.14	4.46	0.62	4.52
29	7.03	7.30	0.73	7.37
98	7.78	10.62	0.31	6.25
108	3.10	3.68	0.33	2.73
113	6.41	7.20	0.47	5.59
117	1.27	1.53	0.16	1.08
134	9.75	12.47	0.91	8.14
147	6.37	7.12	1.09	6.33
192	16.75	18.37	1.35	14.85
222	18.02	18.56	1.10	14.19
224	18.04	19.83	1.17	14.63
244	8.40	9.61	0.63	6.78
255	9.00	9.92	0.98	7.91
266	7.92	9.26	0.74	7.35
273	14.39	12.92	1.55	12.12
335	15.50	17.29	1.07	13.32
364	15.80	12.47	1.16	12.76
162	7.02	7.18	0.55	6.08
212	3.59	2.84	0.25	2.88
294	2.29	2.80	0.11	2.28
360	4.48	4.80	0.29	3.32
387	3.27	3.58	0.28	2.59

n= 32

5. Determination of hGK-1 concentrations in serum samples.

243 samples with no documented clinical back-ground data but consisting of a mixture of normal, BPH and CAP specimens were assayed with hGK-1 assay (Figure 4) and the

- 5 results were compared with the PSA + hGK-1 assay in which the blocking monoclonal anti-PSA antibody A5 that did not cross-react with hGK-1 had been omitted. The samples were classified in four different categories based on their  
 10 PSA-T concentration. The mean of the PSA-T + hGK-1 concentration was calculated as well as the mean proportion (%) of hGK-1 contained in the samples as measured by the hGK-1 assay (Table 3). According to the results the proportion of hGK-1 contained in the samples relative to that of PSA-T ranges from 1 - 3 % although a slight  
 15 percentual increase is observed at higher PSA-T concentrations.

Table 3 Determination of hGK-1 concentrations in serum

PSA-T range, µg/L	n	PSA-T + hGK-1		% of hGK-1	
		mean, µg/L	sd, µg/L	mean, %	sd,
1 - 4	40	2.0	0.75	1.0	2.9
4 - 10	89	6.3	1.7	1.3	3.6
10 - 50	69	21.9	10.2	2.1	3.9
> 50	35	311.5	600.0	3.0	3.1

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of

- 20 embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Lilja, Hans  
Lundwall, Ake  
Lovgren, Janita

(ii) TITLE OF INVENTION: Early Detection of Prostate Cancer (CAP)  
by Employing Prostate Specific Antigen (PSA) and Human  
Glandular Kallikrein (HGK-1)

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Venable, Baetjer, Howard & Civiletti  
(B) STREET: 1201 New York Avenue, NW, Suite 1000  
(C) CITY: Washington  
(D) STATE: DC  
(E) COUNTRY: USA  
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/394,033  
(B) FILING DATE: 24-FEB-1995  
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION (FOR PRIOR APPLICATION):

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(B) REGISTRATION NUMBER: 28,957  
(C) REFERENCE/DOCKET NUMBER: 23635-113884

(viii) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 202-962-4810  
(B) TELEFAX: 202-962-8300

(ix) INFORMATION FOR SEQ ID NO:1:

20

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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## (2) INFORMATION FOR SEQ ID NO:2:

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- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGGTAGCAC CGGTTGGGA TTCCTAGGTA T

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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATGGATCCC ATGTGGGACC TGGTTCTC

28

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGGTAGCGTC GGTTGGGGAT TCTTAGGTAT

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCTACGACT CATCCTGA

18

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

23

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTTAGACCA GATCAAG

17

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Homo sapiens*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCTACAACA CTTCCAG

17

## CLAIMS

1. A bioaffinity assay of prostate-specific antigen (PSA) comprising the measurement of either the concentration of total PSA (PSA-T), the concentration of free form of PSA (PSA-F) or the concentration of PSA complexed to alpha-1-  
5 antichymotrypsin (PSA-ACT), PSA-T being the sum of PSA-F and PSA-ACT, said method additionally comprising the measurement of the concentration of human glandular kallikrein (hGK-1), wherein the concentrations of PSA-T and hGK-1 can be measured in one single assay or in  
10 separate assays, and the sum of the concentrations of PSA-T and hGK-1 is used to determine the ratio
  - a)  $\text{PSA-F}/(\text{PSA-T} + \text{hGK-1})$  and/or
  - b)  $\text{PSA-ACT}/(\text{PSA-T} + \text{hGK-1})$ .
2. A bioaffinity assay according to claim 1 wherein the  
15 ratio  $\text{PSA-F}/(\text{PSA-T} + \text{hGK-1})$  is used to differentiate patients with cancer of the prostate (CAP) from patients with benign prostatic hyperplasia (BPH) or normal male subjects without CAP.
3. A bioaffinity assay according to claim 1 wherein the  
20 ratio  $\text{PSA-ACT}/(\text{PSA-T} + \text{hGK-1})$  is used to differentiate patients with cancer of the prostate (CAP) from patients with benign prostatic hyperplasia (BPH) or normal male subjects without CAP.
4. A bioaffinity assay according to claim 1 wherein the sum  
25 of the concentrations of PSA-T and hGK-1 is measured simultaneously by using antibodies or genetically constructed antibody fragments that recognize common epitopes on both PSA and hGK-1.
5. A bioaffinity assay according to claim 4 wherein a non-  
30 competitive bioaffinity assay is used employing at least two different monoclonal antibodies, polyclonal antibodies,

a combination of monoclonal and polyclonal antibodies or genetically constructed antibody fragments.

6. A bioaffinity assay according to claim 1 wherein the concentrations of PSA-T and hGK-1 are measured separately  
5 after which the sum of the amount of PSA-T and hGK-1 is calculated.

7. A bioaffinity assay according to claim 6 wherein the concentration of hGK-1 is measured by employing two different monoclonal antibodies or antibody fragments in a  
10 non-competitive assay and a third monoclonal antibody or antibody fragment to block the reaction of PSA-T with the two monoclonal antibodies or antibody fragments used in the non-competitive assay.

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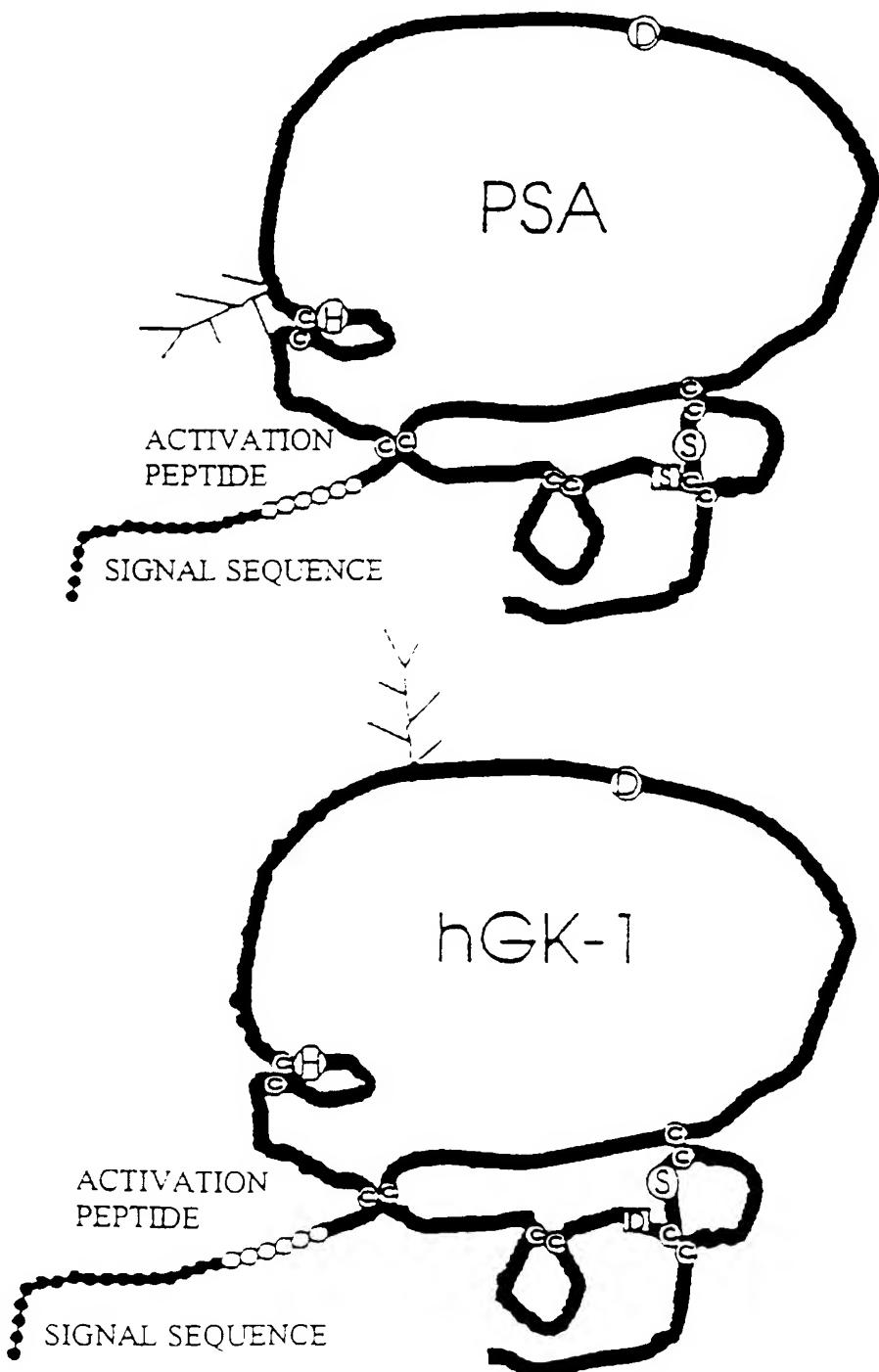


FIG. 1

**SUBSTITUTE SHEET (RULE 26)**

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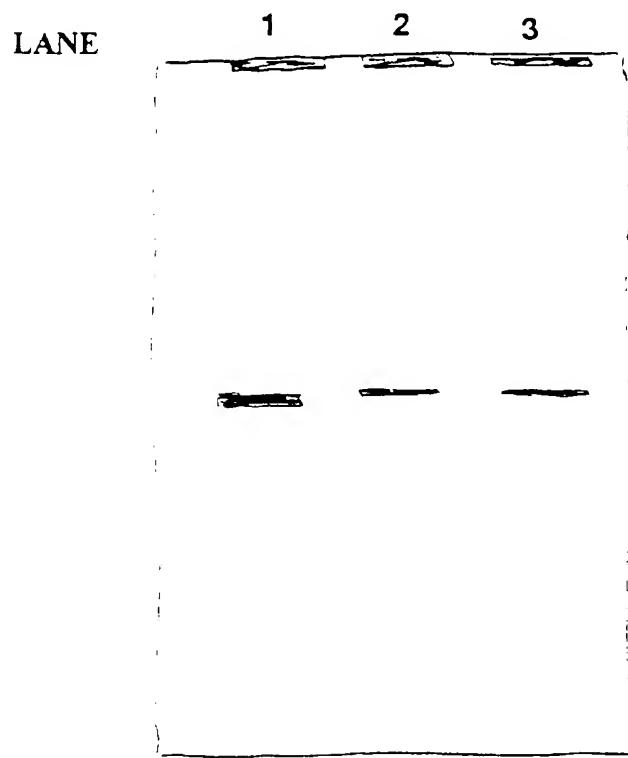
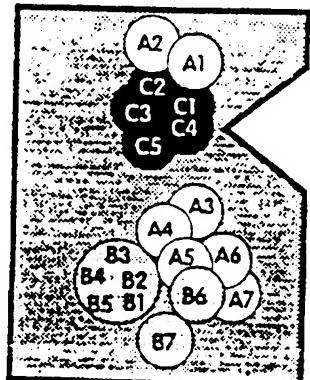
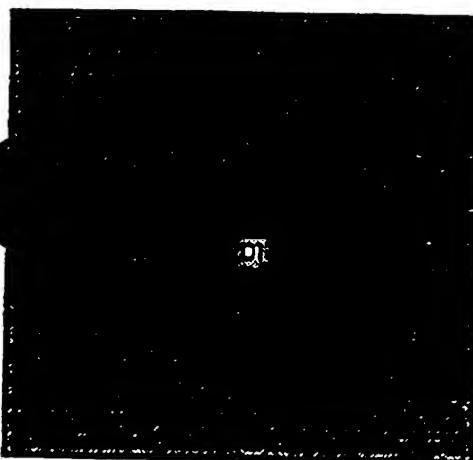
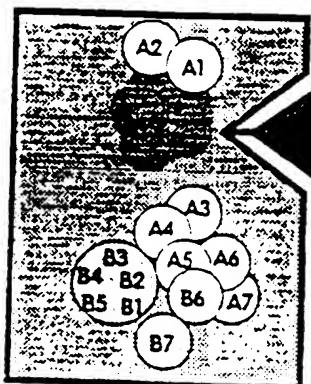


FIG. 2

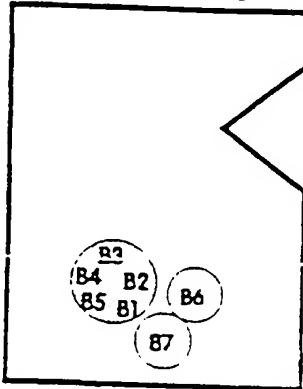
SUBSTITUTE SHEET (RULE 26)

**PSA**

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**ACT****PSA****hGK-1**

Mab defining an epitope accessible on:



- purified PSA and PSA-ACT complex (PSA-T)
- purified PSA (PSA-F) but not on PSA-ACT complex
- rec-hGK-1, purified PSA and PSA-ACT (hGK-1+PSA-T)
- PSA-ACT complex (PSA-C) and purified ACT

FIG. 3

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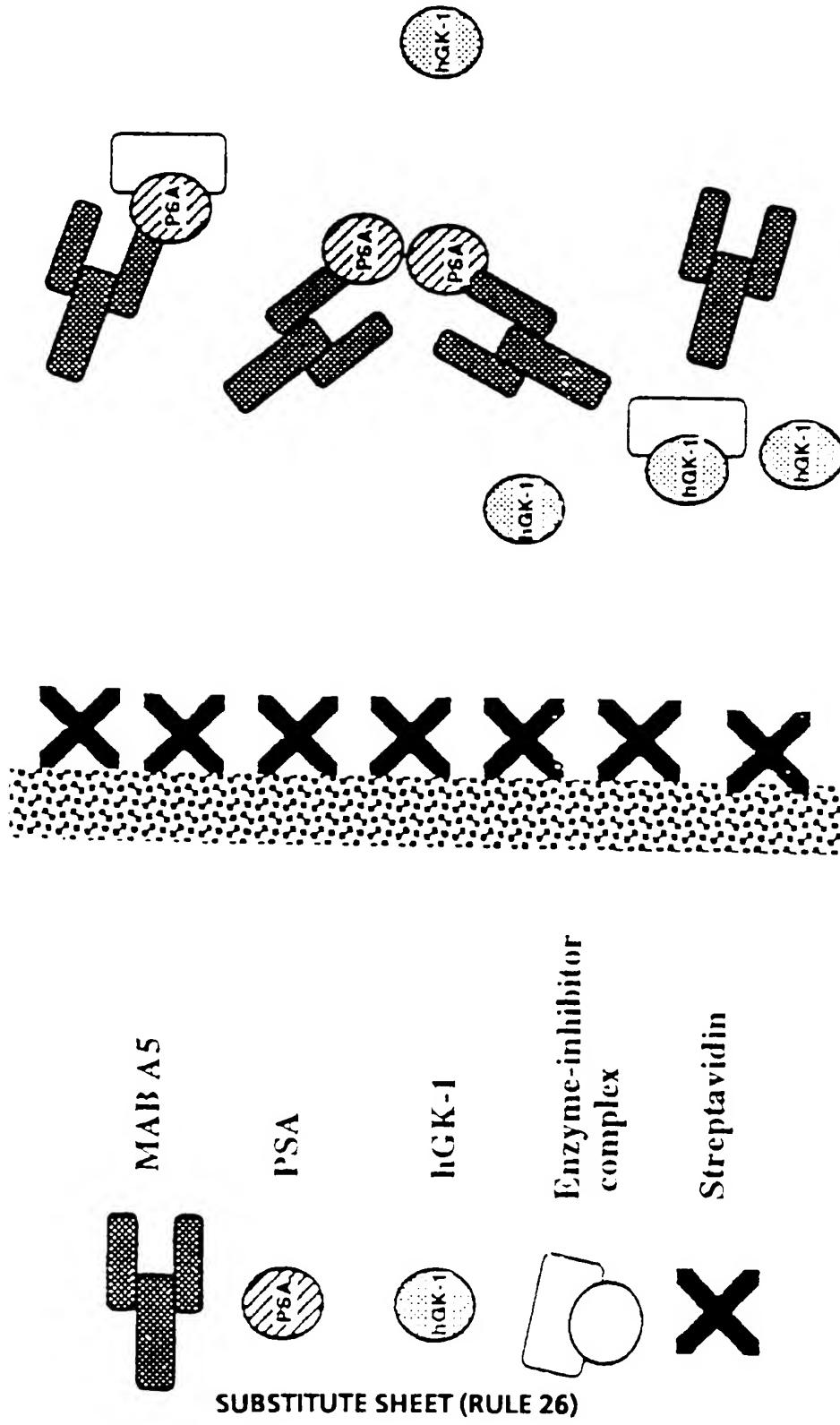


FIG. 4A

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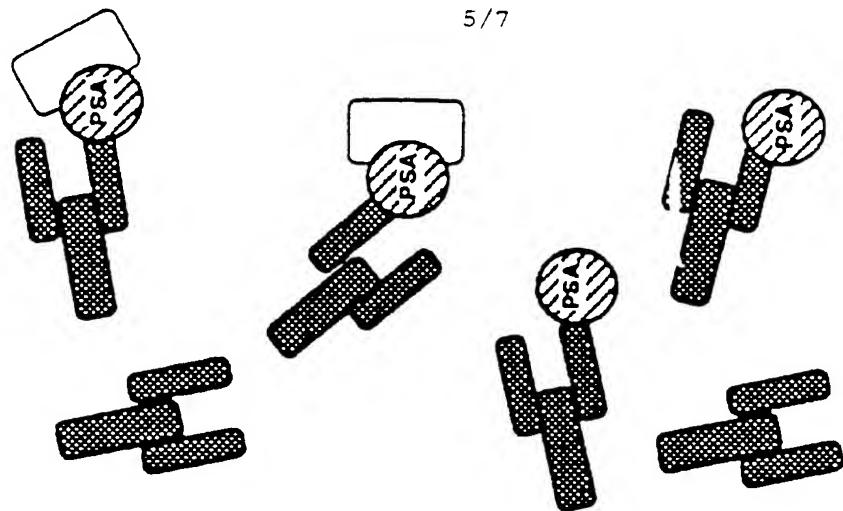
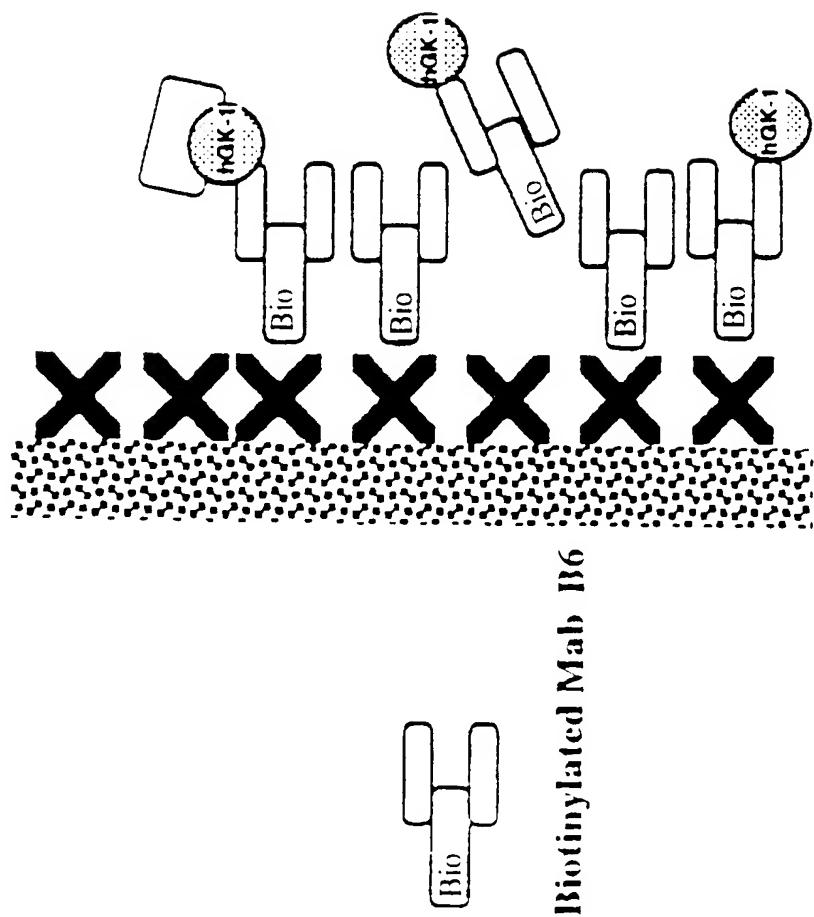


FIG. 4B



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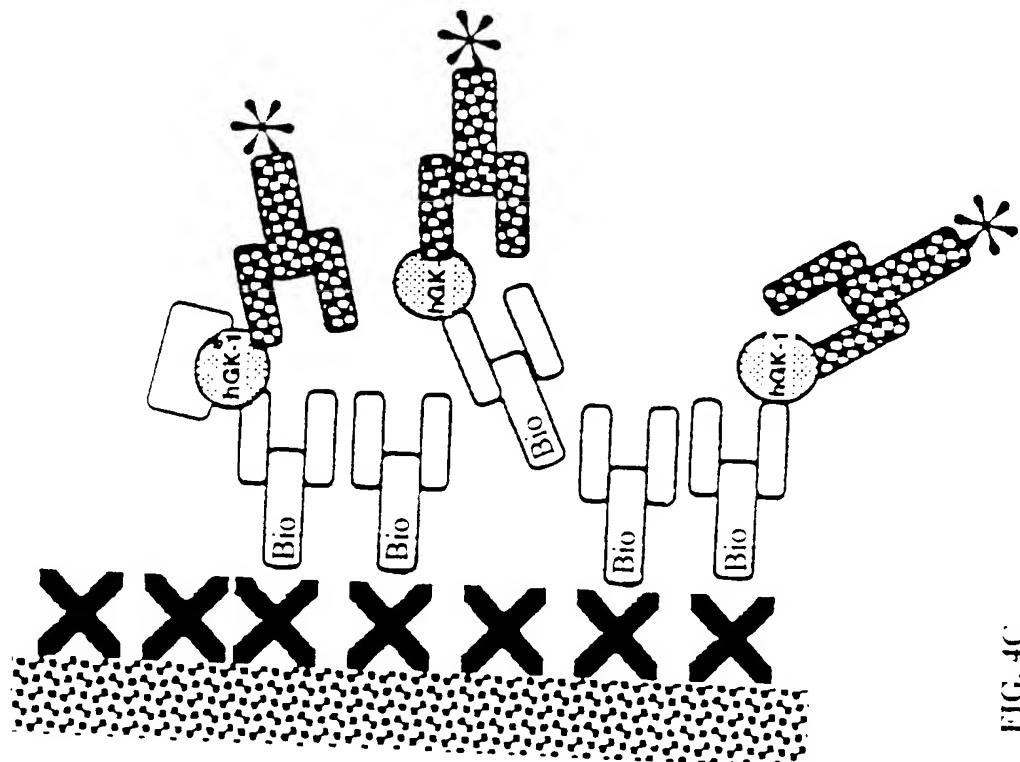


FIG. 4C

Labelled Mab B1



SUBSTITUTE SHEET (RULE 26)

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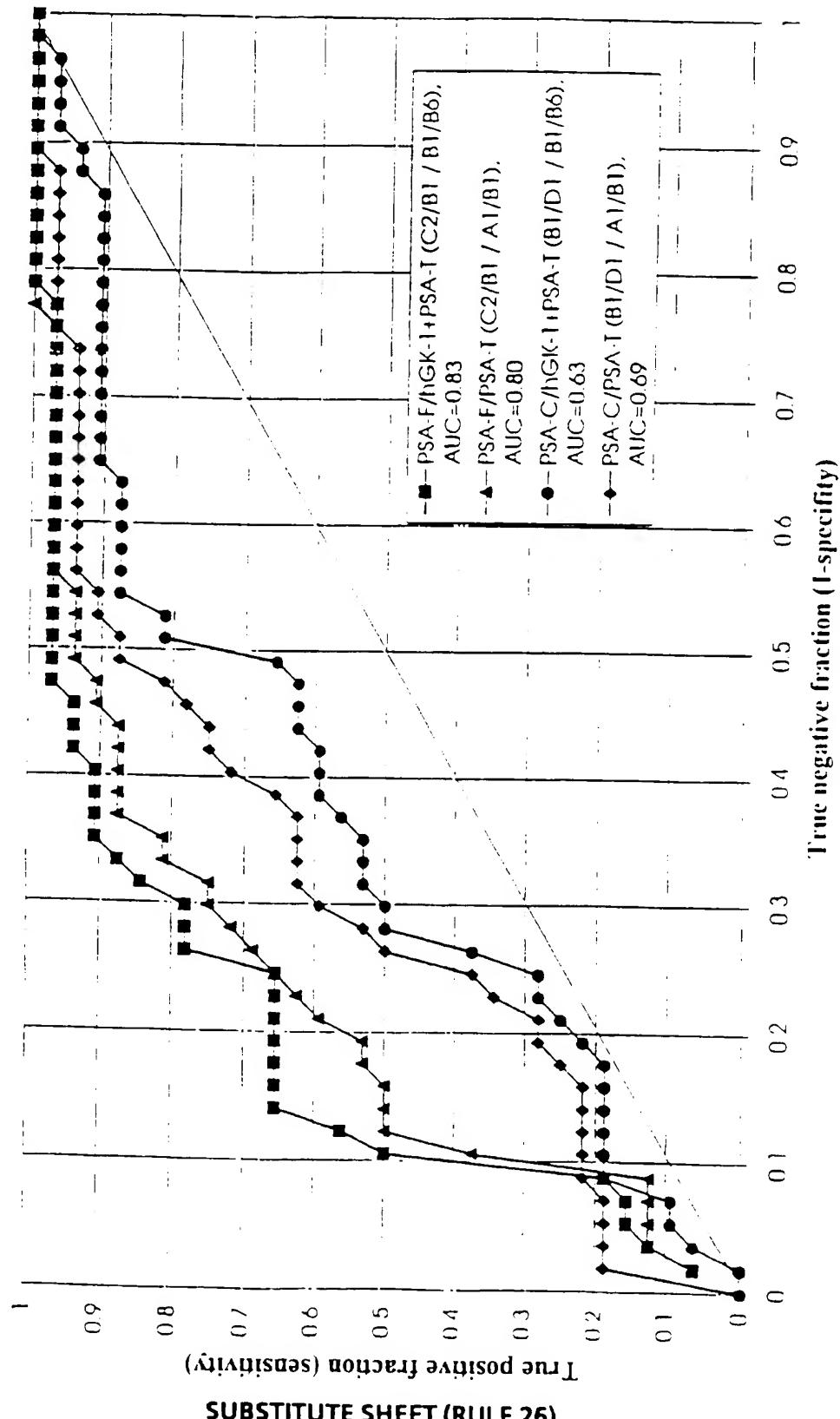


FIG. 5

## INTERNATIONAL SEARCH REPORT

1

International application No.  
PCT/FI 96/00081

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/574, G01N 33/573, C07K 16/30

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CANCERLIT, MEDLINE, WPI, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 9530758 A1 (MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH), 16 November 1995 (16.11.95), page 4, line 20 - line 31  --	1-7
A	National Library of Medicine, file Medline, Medline accession no. 94354993, Henttu P. et al: "Prostate-specific antigen and human glandular kallikrein: two kallikreins of the human prostate", Ann Med 1994 Jun;26(3):157-64  --	1-7
A	WO 9503334 A1 (MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH), 2 February 1995 (02.02.95)  --	1-7

 Further documents are listed in the continuation of Box C. See patent family annex.

## \* Special categories of cited documents

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" other document but published on or after the international filing date
- "C" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "D" document referring to an oral disclosure, use, exhibition or other means
- "E" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

31 May 1996

Date of mailing of the international search report

11-06-1996

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## INTERNATIONAL SEARCH REPORT

2

International application No.  
PCT/FI 96/00081

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0160228 A1 (CETUS CORPORATION), 6 November 1985 (06.11.85)  --	1-7
A	Molecular and Cellular Endocrinology, Volume 99, 1994, Judith A. Clements, "The human kallikrein gene family: a diversity of expression and function"  --	1-7
A	WO 9201936 A1 (LILJA, HANS), 6 February 1992 (06.02.92)  --	1-7
A	The Journal of Urology, Volume 150, July 1993, Anders Christensson et al, "Serum Prostate Specific Antigen Complexed to alpha-Antichymotrypsin as an Indicator of Prostate Cancer" page 100 - page 105  -- -----	1-7

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

01/04/96

International application No.	
PCT/FI 96/00081	

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9530758	16/11/95	NONE		
WO-A1- 9503334	02/02/95	NONE		
EP-A1- 0160228	06/11/85	AU-B,B- 583818 AU-A- 4084585 CA-A- 1252388 JP-A- 60228962 US-A- 4690890		11/05/89 10/10/85 11/04/89 14/11/85 01/09/87
WO-A1- 9201936	06/02/92	AT-T- 134448 DE-T- 540573 DE-U- 9117047 DE-D- 69117292 EP-A,A,B 0540573 ES-T- 2070107 JP-T- 6502719 US-A- 5501983		15/03/96 28/09/95 18/05/95 00/00/00 12/05/93 01/06/95 24/03/94 26/03/96